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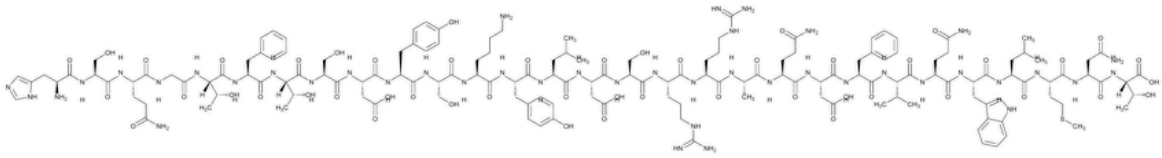
Glucagon

HSQGTFTSDY SKYLDSRRAQ DFVQWLMNT



$C_{153}H_{225}N_{43}O_{49}S$ 3482.80

Glucagon (human) CAS RN®: 16941-32-5; UNII: 76LA80IG2G.

DEFINITION

Glucagon is a peptide hormone that has the property of increasing the concentration of glucose in the blood. It has the same structure (29 amino acids) as the hormone produced by the α-cells of the human pancreas. Glucagon is produced from either synthetic or microbial processes using recombinant DNA (rDNA) technology. When produced by microbial processes, the host cell-derived protein content and/or the host cell-derived or vector-derived DNA content are determined by validated methods. During the course of product development, it must be demonstrated that the manufacturing process produces Glucagon having a biological activity of NLT 0.80 USP Units/mg, using a validated bioassay approved by a competent authority. It contains NLT 93.0% and NMT 105.0% of glucagon ($C_{153}H_{225}N_{43}O_{49}S$) of synthetic origin, calculated on the anhydrous, acetic acid-, ammonium-, and chloride-free basis; and NLT 90% and NMT 105% of glucagon ($C_{153}H_{225}N_{43}O_{49}S$) of recombinant DNA origin, calculated on the anhydrous basis.

IDENTIFICATION

• **A.**

Solution A, Solution B, Mobile phase, System suitability solution, Standard solution, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.

Identity sample solution: Mix equal volumes of the *Standard solution* and the *Sample solution*.

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, and the major peaks of the *Identity sample solution* elute as a single peak.

ASSAY

• **Procedure**

Solution A: Dissolve 16.3 g of potassium phosphate, monobasic in 750 mL of water, adjust with phosphoric acid to a pH of 2.7 (±0.05), add water to 800 mL, add 200 mL of acetonitrile, and degas.

Solution B: Prepare a degassed solution of acetonitrile and water (4:6).

Mobile phase: See *Table 1*.

[Note—The ratio of *Solution A* to *Solution B* can be adjusted to obtain a retention time of about 21 min for the main peak.]

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	61	39
25[a]	61	39

Time (min)	Solution A (%)	Solution B (%)
29	12	88
30	12	88
31	61	39
45	61	39

[a] The end time of the isocratic elution can be adjusted so that the gradient begins after the fourth desamido peak elutes (relative retention time of 1.4). The rest of the program is then adjusted accordingly with this offset.

System suitability solution: Reconstitute a vial of USP Glucagon (Human) RS in 0.01 N hydrochloric acid to obtain a solution having a concentration of about 0.5 mg/mL. Let stand at 50° for 48 h. Prolong the incubation period if necessary. At least 7% total of all four desamido glucagons [(Glu^{3})-glucagon, (Asp^{28})-glucagon, (Glu^{24})-glucagon, and (Glu^{20})-glucagon] should be present in the solution.

Standard solution: Reconstitute a vial of USP Glucagon (Human) RS in 0.01 N hydrochloric acid to obtain a solution having a concentration of about 0.5 mg/mL.

Sample solution: 0.5 mg/mL of Glucagon in 0.01 N hydrochloric acid

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: UV 214 nm

Column: 3-mm × 15-cm; 3-µm packing L1

Temperatures

Autosampler: 2°–8°

Column: 45°

Flow rate: 0.5 mL/min

Injection volume: 15 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Resolution: Four peaks eluting after the glucagon peak that correspond to the desamido glucagons [(Glu^{3})-glucagon, (Asp^{28})-glucagon, (Glu^{24})-glucagon, and (Glu^{20})-glucagon] are clearly visible. The resolution between the main peak and the first eluting desamido peak [(Glu^{3})-glucagon] is NLT 1.5, *System suitability solution*

Tailing factor: NMT 1.8 for the glucagon peak, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glucagon ($C_{153}H_{225}N_{43}O_{49}S$) in the portion of Glucagon taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of the *Standard solution* (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria

For glucagon of recombinant DNA origin: 90%–105% on the anhydrous basis

For glucagon of synthetic origin: 93.0%–105.0% on the anhydrous, acetic acid-, ammonium-, and chloride-free basis

PRODUCT-RELATED SUBSTANCES AND IMPURITIES

• **Procedure**

Solution A, Solution B, Mobile phase, System suitability solution, Standard solution, Sample solution, Chromatographic system, and

System suitability: Proceed as directed in the *Assay*.

Analysis

Sample: *Sample solution*

Calculate the percentage of each impurity in the portion of Glucagon taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for each impurity

r_T = sum of the responses of all peaks

Acceptance criteria

See *Table 2* for glucagon of recombinant DNA origin and *Table 3* for glucagon of synthetic origin. The reporting threshold is 0.05%.

Table 2

Name	Acceptance Criteria, NMT (%)
Total of all four desamido glucagons[a]	2.0
Total impurities and related compounds	6.0

[a] These are (Glu^3)-glucagon, (Asp^{28})-glucagon, (Glu^{24})-glucagon, and (Glu^{20})-glucagon.

Table 3

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Sum of Met(O^{27})-glucagon peaks	0.25 and 0.27 (diastereomers of the sulfoxides)	0.5
(Glu^3)-glucagon	1.09	0.5
(Asp^{28})-glucagon	1.15	0.5
(Glu^{24})-glucagon	1.34	0.5
(Glu^{20})-glucagon	1.39	0.5
Each unspecified impurity	—	0.5
Total impurities	—	2.0

[NOTE—The manufacturer should determine the suitability of the monograph method for their process-related impurities, in particular for an unknown peak eluting at a relative retention time of 0.97, for (D-His^1)-glucagon and (des-Thr^5)-glucagon.]

PROCESS-RELATED IMPURITIES AND OTHER COMPONENTS

[NOTE—These tests only need to be performed for glucagon of synthetic origin.]

Change to read:

• **ACETIC ACID IN PEPTIDES**

Mobile phase: 40 mM sodium hydroxide in water

Standard stock solution: 1.6 mg/mL of potassium acetate (equivalent to 1 mg/mL of acetic acid) in water

Standard solutions: Dilute the *Standard stock solution* with water to obtain 5 solutions with concentrations equivalent to 50, 30, 10, 5, and 1 µg/mL of acetic acid.

Sample solution: 3 mg/mL of Glucagon in *Mobile phase*

[NOTE—If the test solution is not clear, sonicate it for 30–60 min, and pass it through a disposable syringe filter.]

Blank: *Mobile phase*

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: IC

Detector: Conductivity with electrochemical suppressor

Columns

Guard: 4.0-mm × 5-cm; packing L112

Analytical: 4.0-mm × 25-cm; 8.5-µm packing L31

Column temperature: Ambient

Flow rate: 1.0 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solutions* and *Blank*

Suitability requirements

Blank interference: No interfering peak in the elution region of acetate, *Blank*

Peak area drift: Within ±20%, ratio of the acetic acid concentration determined by the multilevel calibration to the theoretical concentration, 10-µg/mL *Standard solution*

Coefficient of determination (R^2): NLT 0.9900 from the standard curve, *Standard solutions*

Relative standard deviation: NMT 5.0% for 6 replicate injections of the 10-µg/mL *Standard solution*

Tailing factor: 0.8–1.6, 10-µg/mL *Standard solution*

Analysis

Samples: *Standard solutions* and *Sample solution*

Calculate the concentration (C_S) of acetic acid, in µg/mL, in each of the *Standard solutions*:

$$\text{Result} = C_{SPA} \times F$$

C_{SPA} = concentration of potassium acetate in each of the *Standard solutions*▲(µg/mL)▲ (ERR 1-Jan-2024)

F = conversion factor for potassium acetate to acetic acid, 0.6119

Construct a calibration curve by plotting the peak responses from the *Standard solutions* versus the concentration of acetic acid (C_S).

Determine the concentration of acetic acid in the *Sample solution* (C_U), in µg/mL, using the quadratic regression.

Determine the percentage of acetic acid in the portion of Glucagon taken:

$$\text{Result} = (C_U \times V_U \times 100)/(W_U \times F)$$

C_U = concentration of acetic acid in the *Sample solution*, determined from the multilevel calibration (µg/mL)

V_U = volume of the *Sample solution* (mL)

W_U = weight of Glucagon used to prepare the *Sample solution* (mg)

F = conversion factor from mg to µg, 1000

Acceptance criteria: NMT 1.2%

Change to read:

• **AMMONIUM**

Mobile phase: 18 mM methanesulfonic acid in water

Standard stock solution: 3 mg/mL of ammonium chloride (equivalent to 1 mg/mL of ammonium) in water

Standard solutions: Dilute the *Standard stock solution* with water to make 5 *Standard solutions* with ammonium concentrations of 10, 5, 2.5, 1, and 0.5 µg/mL.

Sample solution: 0.6 mg/mL of Glucagon in *Mobile phase*

[NOTE—If the test solution is not clear, sonicate it for 30–60 min, and pass it through a disposable syringe filter.]

Blank: *Mobile phase*

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: IC

Detector: Conductivity with electrochemical suppressor

Columns

Guard: 4.0-mm × 5-cm; packing L106

Analytical: 4.0-mm × 25-cm; 8.5-µm packing L106

Column temperature: Ambient

Flow rate: 1.0 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solutions* and *Blank*

Suitability requirements

Blank interference: No interfering peak in the elution region of ammonium, *Blank*

Peak area drift: Within ±20%, ratio of the ammonium concentration determined by the multilevel calibration to the theoretical concentration, 2.5-µg/mL *Standard solution*

Coefficient of determination (R^2): NLT 0.9900 from the standard curve, *Standard solutions*

Relative standard deviation: NMT 5.0% for 6 replicate injections of the 2.5-µg/mL *Standard solution*

Tailing factor: 0.8–2.5, 2.5-µg/mL *Standard solution*

Analysis

Samples: *Standard solutions* and *Sample solution*

Calculate the concentration of ammonium (C_S), in µg/mL, in each of the *Standard solutions*:

$$\text{Result} = C_{SAC} \times F$$

C_{SAC} = concentration of ammonium chloride in each of the *Standard solutions*▲(µg/mL)▲ (ERR 1-Jan-2024)

F = conversion factor from ammonium chloride to ammonium, 0.3372

Construct a calibration curve by plotting the peak responses from each of the *Standard solutions* versus the concentration of ammonium (C_S). Determine the concentration of ammonium in the *Sample solution* (C_U), in µg/mL, using the quadratic regression.

Calculate the percentage of ammonium in the portion of Glucagon taken:

$$\text{Result} = (C_U \times V_U \times 100)/(W_U \times F)$$

C_U = concentration of ammonium in the *Sample solution*, determined from the multilevel calibration (µg/mL)

V_U = volume of the *Sample solution* (mL)

W_U = weight of Glucagon used to prepare the *Sample solution* (mg)

F = conversion factor from mg to µg, 1000

Acceptance criteria: NMT 1.2%

• **Chloride Content:** NMT 4.0%

(See *Titrimetry ⟨541⟩*.)

SPECIFIC TESTS

• **Peptide Mapping**

(See *Biotechnology-Derived Articles—Peptide Mapping ⟨1055⟩*.)

[Note—This test needs to be performed only on material of recombinant DNA origin.]

Determine the peptide fragments, using the following peptide mapping procedure.

Ammonium bicarbonate buffer: Prepare a 1 M ammonium bicarbonate solution, and adjust with ammonia TS to a pH of 10.3. Prepare a mixture of 1 M ammonium bicarbonate and water (1:9).

Enzyme solution: 2 mg/mL of α-chymotrypsin (peptide mapping grade) in *Ammonium bicarbonate buffer*

Solution A: Prepare a degassed mixture of 0.5 mL of trifluoroacetic acid and 1000 mL of water.

Solution B: Prepare a degassed mixture of 0.5 mL of trifluoroacetic acid, 600 mL of ethanol, and 400 mL of water.

Mobile phase: See *Table 4*.

Table 4

Time (min)	Solution A (%)	Solution B (%)
0	100	0
35	53	47
45	0	100
46	100	0
75	100	0

Standard digest solution: Prepare a 5-mg/mL solution of USP Glucagon (Human) RS in 0.01 M hydrochloric acid. Mix 200 µL of this solution with 800 µL of *Ammonium bicarbonate buffer*. To this solution add 25 µL of *Enzyme solution*, and place in a closed vial at about 37° for 2 h. Remove the vial, and stop the reaction immediately by adding 120 µL of glacial acetic acid.

Sample digest solution: Prepare a 5-mg/mL solution of Glucagon in 0.01 M hydrochloric acid. Mix 200 µL of this solution with 800 µL of *Ammonium bicarbonate buffer*. To this solution add 25 µL of *Enzyme solution*, and place in a closed vial at about 37° for 2 h. Remove the vial, and stop the reaction immediately by adding 120 µL of glacial acetic acid.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.0-mm × 5-cm; 5-µm or finer packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard digest solution* and *Sample digest solution*

Suitability requirements

Chromatogram similarity: The chromatogram from the *Sample digest solution* shows a peak pattern similar to that of the chromatogram from the *Standard digest solution*. The peak pattern varies with respect to the purity of the chymotrypsin used for the digestion. If the chymotrypsin is very pure without trypsin, a chromatogram with 4 main peaks instead of 5 will be generated. Additional peptides due to minor cleavages may be seen, but where present, the pattern between the sample and reference is not different.

[Note—The chromatogram from the USP Glucagon (Human) RS certificate is provided as an example to demonstrate the assignment of the predominant cleavage fragments.]

Analysis

Samples: *Standard digest solution* and *Sample digest solution*

Acceptance criteria: The chromatographic profile of the *Sample digest solution* corresponds to that of the *Standard digest solution*.

• **Mass Spectral Analysis**

[Note—These tests only need to be performed for glucagon of synthetic origin.]

(See *Mass Spectrometry ⟨736⟩*.)

Acceptance criteria: The monoisotopic mass is 3480.6 ± 0.5 mass units.

• **Microbial Enumeration Tests ⟨61⟩:** The total aerobic microbial count is NMT 10^2 cfu/g.

• **Water Determination ⟨921⟩, *Method I, Method Ic*:** NMT 10%

• **Bacterial Endotoxins Test ⟨85⟩:** The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Glucagon is used can be met. Where the label states that Glucagon must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Glucagon is used can be met.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in airtight containers, protected from light, and store in a freezer.

• **Labeling:** The labeling states that the material is synthetic or of recombinant DNA origin.

• **USP Reference Standards ⟨11⟩**

USP Glucagon (Human) RS

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
GLUCAGON	Julie Zhang Associate Science & Standards Liaison	BIO12020 Biologics Monographs 1 - Peptides

Chromatographic Database Information: Chromatographic Database

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